(FILE 'HOME' ENTERED AT 13:06:37 ON 21 MAY 2003)

FILE 'MEDLINE, CAPLUS, BIOSIS, AGRICOLA' ENTERED AT 13:06:40 ON 21 MAY 2003

L1 4405 S SORBITOL (2N) DEHYDROGENASE
L2 86 S L1 AND GLUCONOBACTER
L3 58 DUP REM L2 (28 DUPLICATES REMOVED)
L4 41 S L3 AND OXYDANS
L5 2 S L4 AND G624
L6 1031 S GLUCONOBACTER (2N) OXYDANS
L7 56 S L6 AND L1
L8 43 DUP REM L7 (13 DUPLICATES REMOVED)
L9 7 S L8 AND NAD
L10 0 S L9 AND (54 OR 54000)
L11 3 S L9 AND (DA OR KDA)

FILE 'STNGUIDE' ENTERED AT 13:09:22 ON 21 MAY 2003

FILE 'MEDLINE, CAPLUS, BIOSIS, AGRICOLA' ENTERED AT 13:10:47 ON 21 MAY 2003

L12 51 S L6 AND (KDA OR DA)
L13 30 DUP REM L12 (21 DUPLICATES REMOVED)
L14 37 S L1 AND (KDA OR DA) AND NAD
L15 16 DUP REM L14 (21 DUPLICATES REMOVED)
L16 493 S L1 AND NAD
L17 22 S L1 AND MANNITOL AND ARABITOL
L18 16 DUP REM L17 (6 DUPLICATES REMOVED)

FILE 'MEDLINE, CAPLUS, BIOSIS, AGRICOLA' ENTERED AT 13:23:54 ON 21 MAY 2003

L19 86 S L1 AND GLUCONOBACTER
L20 14 S L19 AND KDA
L21 7 DUP REM L20 (7 DUPLICATES REMOVED)
L22 39 S L1 AND (KDA OR DA) AND NAD?
L23 18 DUP REM L22 (21 DUPLICATES REMOVED)

L23 ANSWER 17 OF 18 CAPLUS COPYRIGHT 2003 ACS DUPLICATE 11
AN 1994:649160 CAPLUS
DN 121:249160
TI Purification and properties of **NAD**-dependent **sorbitol dehydrogenase** from apple fruit
AU Yamaguchi, Hideaki; Kanayama, Yoshinori; Yamaki, Shohei
CS Laboratory of Horticultural Science, School of Agricultural Sciences, Nagoya, 464-01, Japan
SO Plant and Cell Physiology (1994), 35(6), 887-92
CODEN: PCPHA5; ISSN: 0032-0781
DT Journal
LA English
AB This is the 1st report of the purifn. of **NAD**-dependent **sorbitol dehydrogenase** (I) from a plant source. I was extd. from apple fruit and purified until it appeared as a single polypeptide chain on a gel after SDS-PAGE. From the apparent mol. wt. of 62 **kDa** obtained by SDS-PAGE and that of 120 **kDa** by gel filtration, I appeared to be a homodimer. Max. rates of oxidn. of sorbitol and redn. of fructose were obsd. at pH 9.6 and 6.0, resp. The Km for oxidn. of sorbitol was 40.3 mM and that for redn. of fructose was 215 mM. The max. rate of oxidn. of sorbitol was .apprx.10-fold higher than that of the redn. of fructose. The results of the kinetic anal. strongly suggest that in vivo the enzyme would favor the conversion of sorbitol to fructose over the reverse reaction. None of the divalent cations tested had any effect on the oxidn. of sorbitol by I. The reaction catalyzed by I was not specific for sorbitol and thus other substrates could also be oxidized. Among the tested substrates, EtOH had a particularly high affinity for the enzyme.

(FILE 'HOME' ENTERED AT 08:55:57 ON 28 MAY 2003)

FILE 'MEDLINE, CAPLUS, BIOSIS, AGRICOLA' ENTERED AT 08:56:00 ON 28 MAY 2003

L1 4274 S SORBITOL (1N) DEHYDROGENASE
L2 464 S L1 AND (50 OR 54 OR 52 OR 55)
L3 12 S L2 AND (DA OR KDA)
L4 7 DUP REM L3 (5 DUPLICATES REMOVED)

FILE 'STNGUIDE' ENTERED AT 08:57:03 ON 28 MAY 2003

FILE 'MEDLINE, CAPLUS, BIOSIS, AGRICOLA' ENTERED AT 08:57:54 ON 28 MAY 2003

L5 83 S L1 AND (KDA OR DA)
L6 38 DUP REM L5 (45 DUPLICATES REMOVED)

FILE 'STNGUIDE' ENTERED AT 09:00:14 ON 28 MAY 2003

ANSWER 6 OF 7 CAPLUS COPYRIGHT 2003 ACS

AN 1995:286639 CAPLUS

DN 122:75279

TI Purification of a membrane-bound **sorbitol dehydrogenase** from Gluconobacter suboxydans

AU Choi, Eui-Sung; Lee, Eun-Hae; Rhee, Sang-Ki

CS Applied Microbiology Research Group, Genetic Engineering Research Institute, KIST, P.O. Box 115, Yusong, Taejon, 305-600, S. Korea

SO FEMS Microbiology Letters (1995), 125(1), 45-50
CODEN: FMLED7; ISSN: 0378-1097

PB Elsevier

DT Journal

LA English

AB A **sorbitol dehydrogenase** was purified from the membrane fraction of Gluconobacter suboxydans KCTC 2111 ( = ATCC 621) by chromatog. on CM-, DEAE-, Mono S and Superose 12 columns. The purified enzyme showed a single activity band upon nondenaturing polyacrylamide gel electrophoresis (PAGE) and three subunits of 75, **50** and 14 **kDa** upon SDS-PAGE. When purified prepns. of the enzyme were reconstituted with pyrroloquinoline quinone (PQQ), the specific enzyme activity was significantly increased (up to 9-fold). The absorption spectrum of purified **sorbitol dehydrogenase** in the reduced state exhibited three absorption maxima (417, 522 and 552 nm), which is in accordance with the typical absorption spectrum of cytochrome c. The **50 kDa** subunit appeared as a red band on unstained SDS-gels, suggesting its identity as a cytochrome. Fluorescence spectra of exts. from purified **sorbitol dehydrogenase** showed an excitation max. at 370 nm and an emission max. at 465 nm, which conformed to those of authentic PQQ. The purified enzyme showed a rather broad substrate specificity with significant activity toward D-mannitol (68%) and D-ribitol (70%) as well as D-sorbitol (100%). The PQQ-dependent **sorbitol dehydrogenase** described in this study is clearly different from the FAD-dependent **sorbitol dehydrogenase** from G. suboxydans var. .alpha. IFO 3254 strain in its cofactor requirement and substrate specificity.

L6 ANSWER 18 OF 38 CAPLUS COPYRIGHT 2003 ACS
AN 1999:243793 CAPLUS
DN 131:41357
TI Purification and properties of NAD+-dependent **sorbitol dehydrogenase** from Bacillus fructosus
AU Uwajima, Takayuki
CS Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Machida, 194-0023, Japan
SO Bioscience, Biotechnology, and Biochemistry (1999), 63(3), 573-574
CODEN: BBBIEJ; ISSN: 0916-8451
PB Japan Society for Bioscience, Biotechnology, and Agrochemistry
DT Journal
LA English
AB **Sorbitol dehydrogenase** (EC 1.1.1.14) (I), which catalyzes the NAD-linked interconversion of D-sorbitol and D-fructose, was purified and crystd. from cell-free exts. of B. fructosus grown on D-sorbitol as a sole carbon source. The cryst. enzyme was homogeneous on disc electrophoresis and ultracentrifugation. The mol. wt. was 102 **kDa** by the sedimentation equil. method. I acted specifically on D-sorbitol, and showed an optimum pH at 9.0. The Km values for D-sorbitol and NAD were 1.1 .times. 10-2 and 2.2 .times. 10-4 M, resp. I was inhibited by p-chloromercuribenzoate, Ag+, Hg2+, and Cu2+.

**STIC-ILL**

5583-A37

**From:** Pak, Yong
**Sent:** Wednesday, May 21, 2003 1:30 PM
**To:** STIC-ILL
**Subject:** 09/926,163

dear stic,

please find the following for 09/926,163:

Crystallization and properties of NAD-dependent D-sorbitol dehydrogenase from Gluconobacter suboxydans IFO 3257
AU Adachi, Osao; Toyama, Hirohide; Theeragool, Gunjana; Lotong, Napha; Matsushita, Kazunobu
CS Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, 753-8515, Japan
SO Bioscience, Biotechnology, and Biochemistry (1999), **63(9), 1589-1595**
CODEN: BBBIEJ; ISSN: 0916-8451

Purification and properties of NAD-dependent sorbitol dehydrogenase from apple fruit
AU Yamaguchi, Hideaki; Kanayama, Yoshinori; Yamaki, Shohei
CS Laboratory of Horticultural Science, School of Agricultural Sciences, Nagoya, 464-01, Japan
SO Plant and Cell Physiology (1994), 35(6), 887-92
CODEN: PCPHA5; ISSN: 0032-0781

Crystallization and properties of NADPH-dependent L-sorbose reductase from Gluconobacter melanogenus IFO 3294
AU Adachi, Osao; Ano, Yoshitaka; Moonmangmee, Duangtip; Shinagawa, Emiko; Toyama, Hirohide; Theeragool, Gunjana; Lotong, Napha; Matsushita, Kazunobu
CS Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, 753-8515, Japan
SO Bioscience, Biotechnology, and Biochemistry (1999), **63(12), 2137-2143**
CODEN: BBBIEJ; ISSN: 0916-8451

thank you.

yong pak
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*Biosci. Biotechnol. Biochem.*, 63 (12), 2137–2143, 1999

# Crystallization and Properties of NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294

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*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*
**Department of Chemical and Biological Engineering, Ube National College of Technology, Tokiwadai, Ube 755-8555, Japan*
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Received July 5, 1999; Accepted August 12, 1999

**NADPH-Dependent L-sorbose reductase (SORD, synonimously NADP-dependent D-sorbitol dehydrogenase) was purified and crystallized for the first time from the cytosolic fraction of *Gluconobacter melanogenus* IFO 3294. The enzyme catalyzed oxidoreduction between D-sorbitol and L-sorbose in the presence of NADP or NADPH. Affinity chromatography by a Blue-dextran Sepharose 4B column was effective for purifying the enzyme giving about 770-fold purification with an overall yield of more than 50%. The crystalline enzyme showed a single sedimentation peak in analytical ultracentrifugation, giving an apparent sedimentation constant of 3.8 *s*. Gel filtration on a Sephadex G-75 column gave the molecular mass of 60 kDa to the enzyme, which dissociated into 30 kDa subunit on SDS-PAGE, indicating that the enzyme is composed of 2 identical subunits. Reduction of L-sorbose to D-sorbitol predominated in the presence of NADPH with the optimum pH of 5.0–7.0. Oxidation of D-sorbitol to L-sorbose was observed in the presence of NADP at the optimum pH of 7.0–9.0. The relative rate of L-sorbose reduction was more than seven times higher to that of D-sorbitol oxidation. NAD and NADH were inert for both reactions. D-Fructose reduction in the presence of NADPH did not occur with SORD. Since the reaction rate in L-sorbose reduction highly predominated over D-sorbitol oxidation over a wide pH range, the enzyme could be available for direct enzymatic measurement of L-sorbose. Even in the presence of a large excess of D-glucose and other substances, oxidation of NADPH to NADP was highly specific and stoichiometric to the L-sorbose reduced. Judging from the enzymatic properties, SORD would contribute to the intracellular assimilation of L-sorbose incorporated from outside the cells where L-sorbose is accumulated in huge amounts in the culture medium.**

**Key words:** acetic acid bacteria; NADP-dependent D-sorbitol dehydrogenase; *Gluconobacter melanogenus*; L-sorbose reductase

Several different kinds of NAD(P)-dependent dehydrogenases in carbohydrate metabolism have been purified from the cytosolic fraction of acetic acid bacteria to characterize the membrane-bound dehydrogenases that catalyze the same reaction.[1-8] In oxidative fermentation, as we have proposed,[9] membrane-bound dehydrogenases have a practical importance to produce various oxidation products and most of the enzymes are localized on the outersurface of the cytoplasmic membrane facing to the periplasmic space. The enzyme reactions are coupled to the respiratory chain of the organism without exception and the electrons generated in substrate oxidation are transferred to the terminal oxidase in the cytoplasmic membranes yielding bioenergy. In the cytosolic fraction, on the other hand, various kinds of NAD(P)-dependent dehydrogenases predominate, most of which show the same reaction as the dehydrogenases in the cytoplasmic membranes under different reaction conditions. Oxidation of alcohol, aldehyde, D-glucose, D-fructose, D-mannitol, D-sorbitol, D-gluconate, 2-keto-D-gluconate, glycerol, and so on has been exemplified with individual enzymes from the cytoplasmic membranes as well as from the cytosolic fraction of acetic acid bacteria.[9] As has been advertised in the purification of NAD-dependent D-sorbitol dehydrogenase (NAD-SLDH) in the previous paper,[8] NADP-dependent D-sorbitol dehydrogenase (synonymously NADPH-dependent L-sorbose reductase, abbreviated as SORD in this study) has worth to compare with NAD-SLDH in many respects. NAD-SLDH catalyzes a shuttle reaction between D-sorbitol and D-fructose and does not obey the Bertrand-Hudson rule in sugar alcohol oxidation. On the other hand, SORD catalyzes a shuttle reaction between D-sorbitol and L-sorbose and fits the Bertrand-Hudson rule similar to NAD(P)-dependent D-mannitol dehydrogenases.[7] Two membrane-bound D-sorbitol dehydrogenases yielding L-sorbose catalyze irreversible one way oxidation reaction coupling to the respiratory chain of the organism.[10,11] It

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¶ On leave from the *Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand.*

is interesting to see that one of the two membrane-bound D-sorbitol dehydrogenase contains a covalently bound FAD as the primary coenzyme,[10] while the other uses pyrroloquinoline quinone (PQQ).[11] The membrane-bound L-sorbose yielding D-sorbitol dehydrogenases obey the Bertrand-Hudson rule and is important in vitamin C manufacturing by the Reichstein method. Provided the enzyme occurs outside the organism, in the periplasmic space, the physiological roles of SORD occurring in the cytosolic fraction of the organism remain to be examined. In this study, purification of SORD was tried from the cytosolic fraction of Gluconobacter melanogenus IFO 3294. In spite of its wide distribution through the genus Gluconobacter, no reasonable purification of SORD has been done so far. Thus, the enzyme is purified and crystallized for the first time in this study. Physicochemical and catalytic properties of SORD are discussed with the crystalline enzyme. Availability of SORD for routine use to screen L-sorbose producing strain is also proposed.

## Materials and Methods

*Chemicals.* NAD, NADP, NADH, NADPH, yeast extract, yeast NAD-dependent alcohol dehydrogenase (151 kDa), and D-glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (105 kDa) were kind gifts from Oriental Yeast Co., Tokyo. Blue-dextran Sepharose 4B was prepared by the method of Ryan and Vestling.[12] Other chemicals used were from commercial sources of guaranteed grade unless otherwise stated.

*Microorganisms and culture conditions. G. melanogenus* IFO 3294 was used throughout this study. The culture medium consisted of 10 g of D-sorbitol, 2 g of glycerol, 1 g of yeast extract, and 1 g of Polypepton in 1 liter of tap water. The pH of the medium spontaneously settled to 6.5 when all these ingredients were mixed. A seed culture in 100 ml of the medium in a 500-ml Erlenmeyer flask was made overnight and transferred to 5 liters of a fresh medium in a 10-L table top fermentor and cultivated for another 12 hr. Then, it was transferred to 30 liters of the medium in a 50-L fermentor and cultured overnight. All cultivation was set at 30°C under shaking or vigorous aeration. About 200 g of wet cells were usually harvested from this kind of culture.

*Assay of enzyme activity.* The enzyme activity of SORD was measured by a routine method used for common NAD(P) enzymes by recording the rate of decrease of NADPH at 340 nm with L-sorbose as the substrate at 25°C. The reaction mixture (1 ml) contained 100 $\mu$mol of L-sorbose, 50 $\mu$mol of potassium phosphate (KPB), pH 6.0, 0.1 $\mu$mol of NADPH, and an appropriate amount of enzyme. D-Sorbitol oxidation was measured in a reaction mixture (1 ml) containing 100 $\mu$mol D-sorbitol, 50 $\mu$mol of Tris-HCl, pH 8.0, 0.1 $\mu$mol of NADP, and the enzyme. The rate of increase in absorbance at 340 nm was recorded. One unit of the enzyme activity was defined as the amount of enzyme catalyzing 1.0 $\mu$mol of NADPH oxidation in L-sorbose reduction or 1.0 $\mu$mol of NADPH formation in D-sorbitol oxidation per min under these conditions. A spectrophotometric absorption coefficient of $E^{1\%}_{cm,280\,nm}=10.0$ was tentatively used for protein concentration measurement. The specific activity was defined as units of enzyme activity per milligram of protein.

*Preparation crude extract.* A buffer solution (Buffer A) of KPB, pH 6.0, containing 50 mM D-sorbitol and 5 mM $\beta$-mercaptoethanol was used throughout this work. Cell suspensions were made by suspending about 10 g of wet cells per 10 ml of Buffer A containing 10 mM KPB and passed through a Rannie high pressure laboratory homogenizer (Rannie model Mini-Lab, type 8.30H, Wilmington, MA, USA) at 10,000 psi. After removal of intact cells by a conventional low speed centrifuge, the crude extract was further centrifuged at 68,000 $\times g$ for 90 min and the resulting supernatant was designated as the cell-free extract.

*Polyacrylamide gel electrophoresis (PAGE).* PAGE by a disc gel in the absence of sodium dodecyl sulfate (native PAGE) was done on a 7.5% polyacrylamide and Tris-glycine buffer, pH 8.3, essentially by the method described by Davis.[13] Protein was stained by Coomassie brilliant blue (CBB R-250).

*SDS-polyacrylamide gel electrophoresis (SDS-PAGE).* SDS-PAGE was done on 12.5% (w/v) slab gel by the methods described by Laemmli.[14] Before application, samples were treated with 6% (w/v) SDS and 0.1 mM dithiothreitol at 60°C for 30 min. The following calibration proteins (Bio-Rad, Hercules, CA, U.S.A.) with the indicated molecular masses were used as references: phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), and lysozyme (14.4 kDa).

*Analytical ultracentrifugation.* Analytical ultracentrifugation was done by a Hitachi model SCP85H ultracentrifuge at 20°C throughout measurements. Estimations of the sedimentation coefficient was done by the methods of sedimentation velocity,[15] which was operated by a combination of a Hitachi UV scanner (ABS-7), an absorption scanner, and a UC processor (DA-7).

*Measurement of molecular mass.* Molecular mass of the native enzyme was measured by gel filtration by the method of Andrews[16] on a Sephadex G-75 column (1 × 120 cm) that had been equilibrated with 2 mM KPB. The following marker proteins were used as references: yeast NAD-dependent alcohol dehydrogenase (150 kDa), NADP-dependent D-glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (105 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and cytochrome *c* (12.4 kDa). Elution was done at a flow rate of 0.5 ml/min with 2 mM KPB, and 35-drop fractions were collected and analyzed. Under these conditions, the peak fraction of the individual marker proteins used came out with the following fraction numbers: NADP-dependent D-glucose-6-phosphate dehydrogenase, 21; bovine serum albumin, 25; ovalbu-

min, 35; cytochrome *c*, 39.

*Heat stability and pH stability.* For examination of heat stability, a diluted enzyme solution (80 $\mu$g protein/ml in 2 mM KPB) was used. The enzyme solution (0.1 ml) in a thin glass tube was directly incubated in the presence or absence of 50 mM L-sorbose under different temperatures for 5 min, and chilled in ice water. The remaining enzyme activity was measured with 10 $\mu$l of the heat-treated enzyme solution under the standard assay conditions. For measurement of pH stability, 10 $\mu$l of the enzyme solution (800 $\mu$g protein/ml in 2 mK KPB) was incubated with 90 $\mu$l of various buffer solutions of different pHs for 5 days in a refrigerator. After the incubation, 2.9 ml of 50 mM KPB, pH 6.0, containing 300 $\mu$mol of L-sorbose and 0.1 $\mu$mol of NADPH, were added and mixed by a flash mixer. The solution was immediately transferred into a glass cuvette to measure the enzyme activity in a photometer at 25°C.

## Results and Discussion

*Purification of NADPH-dependent L-sorbose reductase*

Two hundreds grams of wet cell paste of *G. melanogenus* IFO 3294 harvested from 30 liters of the culture medium was used for the starting material. The crude extract was put to a DEAE-cellulose column (2.5 × 30 cm), which had been equilibrated with 2 mM Buffer A. After the column was washed with the same buffer, elution of SORD was done stepwise with Buffer A containing 0.1 M KCl, 0.3 M KCl, and 0.5 M KCl. More than 75% of the total enzyme activity was eluted with the buffer containing 0.3 M KCl. The enzyme fractions were combined (330 ml) and fractionated with ammonium sulfate. The ammonium sulfate was added to 0.4 saturation (22.6 g/100 ml of enzyme solution) and the pH was adjusted to 6.5 with ammonia water before centrifugation at 15,000 × *g* for 20 min. To the supernatant, ammonium sulfate was further added to 0.6 saturation (12.0 g/100 ml of enzyme solution) and the precipitate emerged was collected by centrifugation at 15,000 × *g* for 20 min. About 85% of the total enzyme activity was recovered in the precipitate. The precipitate was dialyzed overnight against 2 mM Buffer A. The dialyzed enzyme was put on a DEAE-Sephadex A-50 column (1.5 × 25 cm), which had been equilibrated with 2 mM Buffer A. After the column was washed with the same buffer containing 0.075 M KCl, a linear gradient chromatography of KCl was done between KCl concentrations of 0.075 M and 0.35 M (500 ml each). The enzyme activity appeared at the KCl concentration around 0.18 M. SORD proteins were collected by ammonium sulfate precipitation at 0.75 saturation (46.5 g/100 ml) and dialyzed against 2 mM KPB containing 5 mM $\beta$-mercaptoethanol. The dialyzed enzyme solution was put on a Blue-dextran Sepharose 4B column (2.5 × 30 cm), which had been equilibrated with the same buffer used for dialysis. The enzyme activity was eluted with the buffer containing 50 mM D-sorbitol and 0.25 M KCl. By this step, contaminating similar enzymes such as NAD-dependent and NADP-dependent D-mannitol dehydrogenases,[7] which are not adsorbed by the Blue-dextran Sepharose 4B column, must be removed from SORD, though exact checking was not done. The enzyme proteins were collected by ammonium sulfate precipitation as above. The precipitate was dissolved in a small amount of the buffer and put on a Sephadex G-200 column (1 × 180 cm), which had been equilibrated with 0.1 M Tris-HCl, pH 7.4, containing 5 mM $\beta$-mercaptoethanol, 10 mM $MgSO_4$, and 10% glycerol. After a highly concentrated enzyme solution was prepared at room temperature in the presence of ammonium sulfate of 0.4 saturation, the enzyme solution was put in a refrigerator overnight. Fine needles of the enzyme crystals appeared. For the growth of enzyme crystals, saturated ammonium sulfate solution was added dropwise after several hours until the top meniscus became clear. The first crystals (crystalline fraction) were collected by a table top centrifuge and the precipitate was dissolved in a small volume of 30 mM KPB, pH 6.0, containing $\beta$-mercaptoethanol. Crystallization was repeated to give fine needles as shown in Fig. 1. SORD was purified about 770-fold from the cell-free extract with an overall yield of 55%. The summary of enzyme purification is shown in Table 1.

**Fig. 1.** Crystals of NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294.
The photomirograph was taken under 148-fold of magnification.

*Physicochemical properties of crystalline enzyme*

When analyzed in analytical ultracentrifugation, SORD showed a single sedimentation peak with an apparent sedimentation coefficient of 3.8 *s* (Fig. 2). The photograph was taken every 25 min due to its small molecular mass giving a diffused meniscus when centrifuged over 80 min. Molecular mass measurement by SDS-PAGE gave an apparent molecular mass of 30 kDa, which was almost the same position to that of carbonic anhydrase (Fig. 3-A). The crystalline enzyme was homogeneous in disc gel electrophoresis, showing a single protein band (Fig. 3-B). When a mixture of SORD and standard marker proteins was put on a Sephadex G-75 column, SORD appeared at the fraction number of 28, which corresponded to an apparent molecular mass of 60 kDa. In an alternative molecular mass measurement by the same method, SORD was mixed with NADP-dependent D-mannitol dehydrogenase of which

**Table 1.** Purification of NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294

| Step | Total protein (mg) | Total activity* (units) | Specific activity (units/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 35350 | 3248 | 0.1 | 100 | 1 |
| DEAE-cellulose | 4050 | 2460 | 0.6 | 76 | 6 |
| DEAE-Sephadex A-50 | 2205 | 2438 | 1.1 | 75 | 11 |
| Bluedextran Sepharose | 320 | 2825 | 8.8 | 87 | 88 |
| Sephadex G-200 | 143 | 2550 | 17.8 | 78 | 178 |
| DEAE-Sephadex A-50 | 55 | 2390 | 43.4 | 73 | 434 |
| Crystalline fraction | 24 | 1860 | 77.0 | 57 | 770 |

* Enzyme activity was assayed in 50 mM KPB, pH 6.0, by measuring the decrease of absorbance of NADPH using L-sorbose as the substrate.

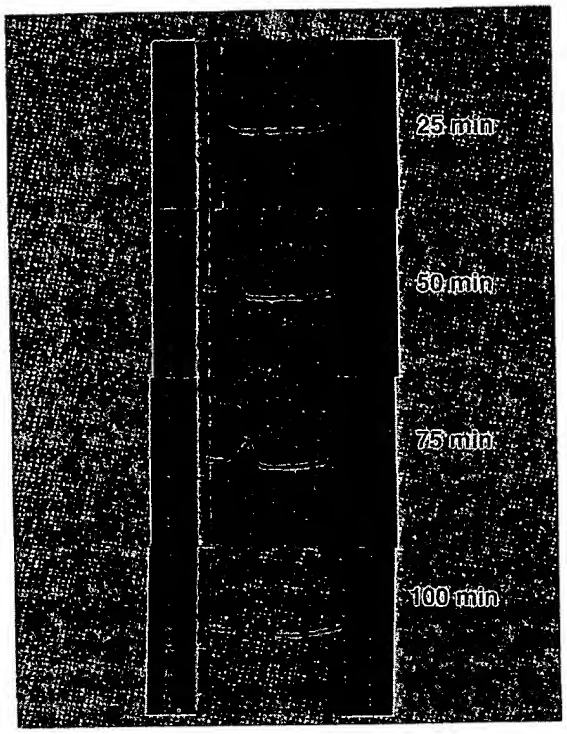


**Fig. 2.** Sedimentation Patterns of Crystalline NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294.
Photographs were taken every 25 min as indicated after reaching 60,000 rpm. The enzyme solution containing 11.8 mg protein/ml in 2 mM KPB, pH 6.0, was used.

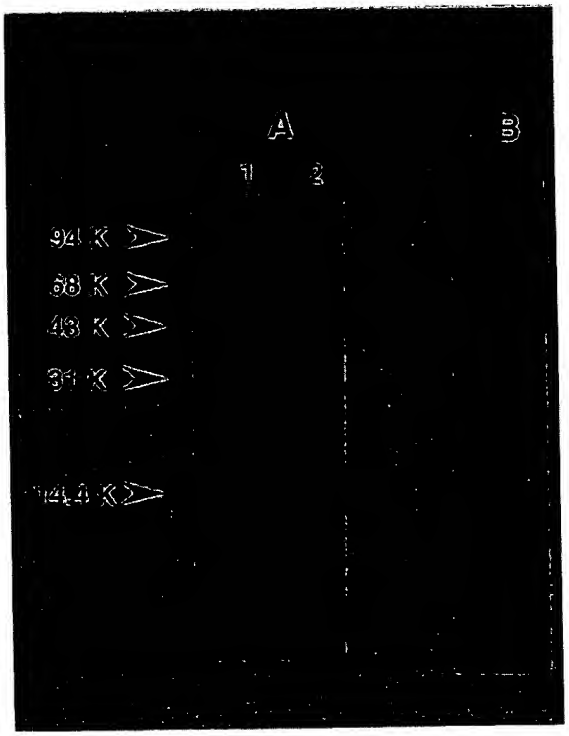


**Fig. 3.** Gel Electrophoresis of Crystalline NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294.
A diluted crystalline enzyme was used. (A) SDS-PAGE. Lane 1, marker proteins; lane 2, SLDH (10 $\mu$g protein). (B) Native gel electrophoresis. Protein (25 $\mu$g) was loaded.

the molecular mass had been measured to be 50 kDa[7] and analyzed under the same conditions as above. When compared the elution profiles of SORD with those of NADP-dependent D-mannitol dehydrogenase, SORD always came out immediately before NADP-dependent D-mannitol dehydrogenase. Thus, the apparent molecular mass of 60 kDa must be probable to SORD. It is also acceptable to conclude that SORD is composed of two identical subunits of 30 kDa.

*Catalytic properties of crystalline enzyme*

L-Sorbose was most rapidly reduced to D-sorbitol at pH 5.0–7.0 in the presence of NADPH, but NADH was inert as the electron donor. Potassium phosphate seemed to be a favorable buffer. McIlvaine buffer also showed the highest enzyme rate at a similar level as shown by potassium phosphate buffer. However, the enzyme activity was repressed at about 60% with acetate buffer compared to those observed with potassium phosphate buffer. D-Sorbitol was oxidized by the enzyme but the relative rate of D-sorbitol oxidation was less than 15% of that of L-sorbose reduction, when assayed with Tris-HCl buffer. It is very much like the case of 5-keto-D-fructose reductase,[6] by which D-fructose is oxidized at lower levels, though keto-D-gluconate reductases[2,3] show a reasonable oxidation for D-gluconate. The effects of buffer species on the enzyme activity of SORD are shown in Fig. 4. Other substrates including various sugars and sugar alcohols were used as substrates for SORD. The enzyme was concluded to be highly specific to L-sorbose reduction, thus the terminology as NADPH-dependent L-sorbose reductase would be better than NADP-dependent D-sorbitol dehydrogenase, as judged from its substrate specificity as shown in Table 2.

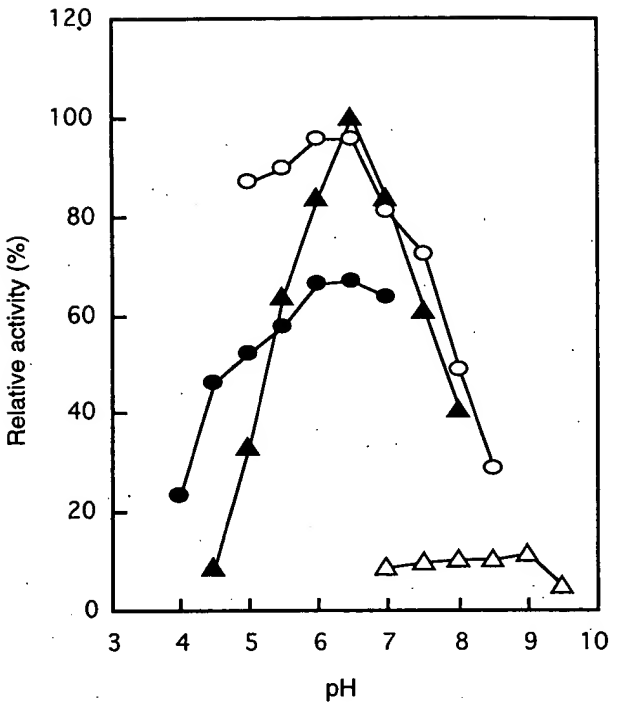


**Fig. 4.** Effects of pH on Crystalline NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294.

Enzyme activity (0.4 $\mu$g of protein for one assay) was measured with different pHs of buffer solution as indicated. (▲), McIlvaine buffer; (●), acetate buffer; (○), potassium phosphate buffer; (△), Tris-HCl buffer. D-Sorbitol oxidation was done with Tris-HCl buffer.

**Table 2.** Substrate Specificity of NADPH-Dependent L-Sorbose Reductase

| Substrate | Polyol dehydrogenase (units/mg protein) | Substrate | Ketose reductase (units/mg protein) |
|---|---|---|---|
| D-Glucose | 0 | D-Fructose | 0 |
| D-Mannose | 0 | L-Sorbose | 77 |
| D-Galactose | 0 | D-Xylulose* | 0 |
| D-Tagatose | 0 | D-Ribulose* | 0 |
| D-Sorbitol | 11 | Dihydroxyacetone | 0 |
| D-Mannitol | 0 | | |
| L-Iditol | 0 | | |
| Dulcitol | 0 | | |
| D-Xylose | 0 | | |
| D-Arabinose | 0 | | |
| D-Arabitol | 0 | | |
| L-Arabitol | 0 | | |
| Xylitol | 0 | | |
| Ribitol | 0 | | |
| Erythritol | 0 | | |
| D-Erythrose | 0 | | |
| Glycerol | 0 | | |

Enzyme activity was measured with the crystalline preparation of SORD under the standard assay conditions as described in the Materials and Methods.

* The final substrate concentration was adjusted to 0.1 M in the reaction mixture (total volume 1 ml), except that D-xylulose and D-ribulose were done with the final concentration of 0.01 M.

There were no data about reduction of L-ribulose and L-erythrulose with SORD, due to unavailability of the substrates from commercial sources. Apparent Km values for L-sorbose and NADPH were measured to be 35 mM and 32 $\mu$M, respectively. As judged from the lower reaction rates for D-sorbitol oxidation by SORD as above, measurements of Km for D-sorbitol and NADP were not done, because there would have no significance. SORD was stable to heating at 45°C for 5 min, however enzyme activity was completely lost when the enzyme was heated for 5 min over 55°C. The thermal stability was not improved in the presence of L-sorbose. The enzyme activity of SORD was stable in solution of pH 5–7.5 for several days when a diluted enzyme solution was stored in a refrigerator (data not shown).

As judged from the catalytic properties examined with SORD, the role of the physiological function of SORD can be assigned to L-sorbose reduction yielding D-sorbitol in the cytoplasm. The following discussion would be accepted reasonably. Acetic acid bacteria produce a huge amount of various oxidation products in the culture medium, as 2-keto-D-gluconate and/or 5-keto-D-gluconate when grown on D-glucose, and as 5-keto-D-fructose when grown on D-fructose.[9] Such novel oxidation products can be thought to be a temporary stock substance which can be used thereafter only by acetic acid bacteria, because acetic acid bacteria have a novel enzyme, 2-keto-D-gluconate reductase[1,2] and/or 5-keto-D-gluconate reductase[3] catalyzing D-gluconate formation which can be used via pentose phosphate pathway of the organisms after incorporated and phosphorylated. 5-Keto-D-fructose is reduced to D-fructose by 5-keto-D-fructose reductase[6] and then further metabolized by the organisms. The enzyme reaction of 5-keto-D-fructose reductase is much intense in the reduction of 5-keto-D-fructose yielding D-fructose, and D-fructose oxidation occurs at very low level even though the assay is made under optimum condition. Likewise, L-sorbose accumulated outside the cells is incorporated into the cells when other usable carbon and energy sources come to be exhausted. L-Sorbose would be converted to D-sorbitol by SORD which can be used as carbon and energy sources.

The effects of other hexoses or pentoses on the reaction rate of L-sorbose reduction were examined with the crystalline enzyme to see whether any disturbance in L-sorbose reduction by such the compounds occurs. When D-glucose, D-fructose, D-mannitol, and D-xylose were present more than 10 times higher than the concentration of L-sorbose in the reaction mixture, no inhibition on L-sorbose reduction was observed (data not shown). This strongly supports an idea that SORD prepared in this study is available for the measurement of L-sorbose in the presence of other sugars or sugar alcohols. Due to lack of good enzymes for enzymatic L-sorbose measurement, HPLC is the only technique at this moment. The only example is the membrane-bound L-sorbose dehydrogenase from *G. melanogenus* UV10 characterized by Sugisawa *et al.*[17] and SORD in this study. If the membrane-bound enzyme were available for the purpose, the enzyme would be useful for the end point measurement, by which a trace of L-sorbose could be assayed with high accuracy. Membrane-bound dehydrogenases can be available for the measurement of individual substrates

**Table 3.** Comparison of Properties among NAD(P)-Dependent Sugar Alcohol Dehydrogenases from *Gluconobacter* strains

| | NADP-MDH[a] | NAD-MDH[b] | NAD-SLDH[c] | NADP-SLDH[d] |
|---|---|---|---|---|
| Molecular mass | 50 kDa | 130 kDa | 98 kDa | 60 kDa |
| Subunit | 50 kDa | ND* | 26 kDa | 30 kDa |
| Numbers of subunits | 1 | ND | 4 | 2 |
| Svedverg unit | 3.6 s | ND | 5.1 s | 3.8 s |
| Substrate | D-mannitol D-fructose | D-mannitol D-fructose | D-sorbitol D-fructose | D-sorbitol L-sorbose |
| *Km* for | | | | |
| D-Mannitol | 10 mM | 20 mM | — | — |
| D-Sorbitol | — | — | 5 mM | ND |
| D-Fructose | 12 mM | 33 mM | 20 mM | — |
| L-Sorbose | — | — | — | 35 mM |
| NAD | — | $2.5\times10^{-4}$ M | $2.1\times10^{-4}$ M | — |
| NADH | — | $1.0\times10^{-5}$ M | $2.8\times10^{-4}$ M | — |
| NADP | $2.5\times10^{-5}$ M | — | — | ND |
| NADPH | $1.9\times10^{-5}$ M | — | — | $3.2\times10^{-5}$ M |
| *Vmax* for | | | | |
| D-Mannitol | 221 μmol/mg | 150 μmol/mg | — | — |
| D-Sorbitol | — | — | 136 μmol/mg | 11 μmol/mg |
| D-Fructose | 235 μmol/mg | 188 μmol/mg | 34 μmol/mg | — |
| L-Sorbose | — | — | — | 77 μmol/mg |
| *Optimum pH* | | | | |
| D-Mannitol | 9.0 | 9.0 | — | — |
| D-Sorbitol | — | — | 9.0–11.0 | 7.0–9.0 |
| D-Fructose | 6.5–7.5 | 6.0 | 5.0–6.0 | — |
| L-Sorbose | — | — | — | 5.0–7.0 |

[a] NADP-Dependent D-mannitol dehydrogenase from *G. suboxydans* IFO 12528.[7]
[b] NAD-Dependent D-mannitol dehydrogenase from *G. suboxydans* IFO 12528.[7]
[c] NAD-Dependent D-sorbitol dehydrogenase from *G. suboxydans* IFO 3257.[8]
[d] NADP-Dependent D-sorbitol dehydrogenase(=NADPH-dependent L-sorbose reductase, SORD) from *G. melanogenus* IFO 3294 (this study).
ND: not determined.

by the end point measurement.[18] These enzymes can measure traces of the substrates by which an absolute measurement of the substrate becomes possible, because the membrane-bound enzymes catalyze a one way oxidation reaction. On the other hand, the rate assay system using NAD(P)-dependent enzymes has still a merit by its simplicity for handling the enzyme in the routine assays. Though they are unable to make an absolute measurement of the substrate, unlike membrane-bound dehydrogenases, they can outdo HPLC by their simplicity and rather higher performance. It has become urgently important to establish an enzymatic measurement for D-fructose, D-sorbitol, D-mannitol, and L-sorbose. The NADP-dependent D-mannitol dehydrogenase was discussed as useful enzyme for D-fructose measurement in our previous paper.[7] It is important for microbial screening to search for a useful strain producing D-fructose. Similarly, SORD must be useful for the screening of L-sorbose producing microorganisms.

Development and application of thermotolerant acetic acid bacteria for oxidative fermentation has attracted strong interest in many respects when compared with nonthermotolerant strains.[19] Screening of thermotolerant acetic acid bacteria that produce L-sorbose and D-fructose has been done successfully using SORD and NADP-dependent D-mannitol dehydrogenase (D. Moonmangmee *et al.*, manuscript in preparation). Occurrence of many different novel dehydrogenases in carbohydrate metabolism is known, as has been reviewed.[9,20] Though Kersters *et al.* have reviewed a wide variety of NAD(P)-linked dehydrogenases in acetic acid bacteria,[21] purification of an enzyme corresponding to SORD has not been done. Hoshino *et al.* reported a novel enzyme, NAD(P)-dependent L-sorbosone dehydrogenase from *G. melanogenus* UV10,[22] however the enzyme is absolutely different from the SORD reported in this paper. Thus, SORD in this paper would be the first successful example in which an enzyme catalyzing L-sorbose reduction to D-sorbitol with high specificity is crystallized. The physicochemical and catalytic properties of SORD were compared with other similar enzymes from *Gluconobacter* strains, since they are useful in enzymatic measurement for D-fructose, D-sorbitol, D-mannitol, and L-sorbose (Table 3). All of these enzymes must be useful for monitoring ketohexoses, ketopentoses, and other related substances.

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Crystallization and properties of NAD-dependent D-sorbitol dehydrogenase from Gluconobacter suboxydans IFO 3257
AU Adachi, Osao; Toyama, Hirohide; Theeragool, Gunjana; Lotong, Napha; Matsushita, Kazunobu
CS Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, 753-8515, Japan
SO Bioscience, Biotechnology, and Biochemistry (1999), **63(9), 1589-1595**
CODEN: BBBIEJ; ISSN: 0916-8451

Purification and properties of NAD-dependent sorbitol dehydrogenase from apple fruit
AU Yamaguchi, Hideaki; Kanayama, Yoshinori; Yamaki, Shohei
CS Laboratory of Horticultural Science, School of Agricultural Sciences, Nagoya, 464-01, Japan
SO Plant and Cell Physiology (1994), 35(6), 887-92
CODEN: PCPHA5; ISSN: 0032-0781

Crystallization and properties of NADPH-dependent L-sorbose reductase from Gluconobacter melanogenus IFO 3294
AU Adachi, Osao; Ano, Yoshitaka; Moonmangmee, Duangtip; Shinagawa, Emiko; Toyama, Hirohide; Theeragool, Gunjana; Lotong, Napha; Matsushita, Kazunobu
CS Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, 753-8515, Japan
SO Bioscience, Biotechnology, and Biochemistry (1999), **63(12), 2137-2143**
CODEN: BBBIEJ; ISSN: 0916-8451

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*Plant Cell Physiol.* 35(6): 887–892 (1994)


# Purification and Properties of NAD-Dependent Sorbitol Dehydrogenase from Apple Fruit

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This is the first report of the purification of NAD-dependent sorbitol dehydrogenase (NAD-SDH) from a plant source. The enzyme was extracted from apple (*Malus domestica* cv. Ourin) fruit and purified until it appeared as a single polypeptide chain on a gel after SDS-PAGE. From the apparent molecular mass of 62 kDa obtained by SDS-PAGE and that of 120 kDa by gel filtration, the enzyme appeared to be a homodimer. Maximum rates of oxidation of sorbitol and reduction of fructose were observed at pH 9.6 and pH 6.0, respectively. The $K_m$ for oxidation of sorbitol was 40.3 mM and that for reduction of fructose was 215 mM. The maximum rate of oxidation of sorbitol was about 10 times higher than that of the reduction of fructose. The results of the kinetic analysis strongly suggest that in vivo the enzyme would favor the conversion of sorbitol to fructose over the reverse reaction. None of the divalent cations tested had any effect on the oxidation of sorbitol by NAD-SDH. The reaction catalyzed by NAD-SDH was not specific to sorbitol and other substrates could also be oxidized. Among the tested substrates, ethyl alcohol had a particularly high affinity for the enzyme.

**Key words:** Apple (*Malus domestica*) — Purification — Sorbitol — Sorbitol dehydrogenase.

Sorbitol is a widely distributed soluble carbohydrate that is found in a number of plants (Lewis and Smith 1967, Washüttl et al. 1973), being particularly abundant in plants of the Rosaceae (Plouvier 1963, Chong 1971, Yamaki et al. 1979, Wallart 1980). As a major product of photosynthesis, that is translocated from leaves (Webb and Burley 1962, Bieleski 1969, 1982, Chong and Taper 1971, Loescher 1987), sorbitol plays an important role in the metabolism of photosynthates in apple (Yamaki 1980, Yamaki and Ishikawa 1986).

Studies of the changes in and the factors that control the activities of enzymes responsible for the synthesis and degradation of photosynthates, which are translocated to sink tissues, could lead to a better understanding of processes that control the storage of photosynthates (Hawker 1971). NAD-dependent sorbitol dehydrogenase (NAD-SDH; EC 1.1.1.14) catalyzes the oxidation of sorbitol and the reduction of fructose (Negm and Loescher 1979). In the developing maize kernel, sucrose arriving from the phloem is metabolized to yield fructose as one of the products (Doehlert 1987). NAD-SDH has been partially purified from maize endosperm and its possible significance in the utilization of fructose has been discussed (Doehlert 1987). NAD-SDH has been detected in apple callus (Negm and Loesher 1979), and seasonal changes in its activity has been reported (Yamaki and Ishikawa 1986). Sorbitol accounts for about 80% of the total soluble carbohydrate in apple leaves, spurs, and peduncles but only about 3% to 8% in the fruit. This difference has been attributed to the high activity of NAD-SDH in the fruit (Yamaki 1986). NAD-SDH allows the utilization of the major translocated carbohydrate, namely sorbitol, for the synthesis of the major sugar that is accumulated, namely fructose, in apple fruit (Knee 1993). These earlier results and observations substantiate the importance of NAD-SDH in the regulation of sorbitol metabolism.

In this report we describe the purification of NAD-SDH from apple fruit and some characteristics of the enzyme.

Abbreviations: NAD-SDH, NAD-dependent sorbitol dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

## Materials and Methods

*Plant material*—Apple (*Malus domestica*, cv. Ourin)

fruits at a pre-climacteric stage were obtained from the Fruit Tree Research Station, Morioka, Iwate, Japan, and stored in a cold storage room ($O_2$, $CO_2$, and humidity were at the ambient levels; temperature was 0°C.) until use.

*Extraction and purification of the enzyme*—The enzyme was extracted in a cold room in which the temperature was maintained at 4°C. A total of 800 g of peeled and cored apple flesh was homogenized in 800 ml of 0.15 M $KH_2PO_4$-NaOH buffer (pH 8.0) that contained 2 mM PMSF, 10 mM Na-L-ascorbate, 10 mM 2-mercaptoethanol, and 80 g of Polyclar AT (Gokyo Sangyo Co., Tokyo, Japan). The homogenate was squeezed through a layer of fine cloth and the filtrate was centrifuged at 13,000 × *g* for 15 min. The supernatant was passed through a column (12.5 cm i.d. × 5.5 cm) of Sephadex G-25 (Pharmacia Co., Uppsala, Sweden) to remove phenolic compounds. The filtrate was brought to 40% saturation with $(NH_4)_2SO_4$, centrifuged at 13,000 × *g* for 20 min, and the pellet was discarded. The supernatant was mixed with 60 ml of Butyl-Toyopearl 650 C (Tosoh Co., Tokyo, Japan), which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) plus $(NH_4)_2SO_4$ to 40% saturation, 0.2 mM PMSF, and 2 mM 2-mercaptoethanol, and the protein was adsorbed to the resin. The Butyl-Toyopearl 650 C with the adsorbed proteins was packed in a column (5 cm i.d. × 3.5 cm) and proteins were eluted with 10 mM Tris-HCl buffer (pH 8.0) that contained 0.2 mM PMSF and 2 mM 2-mercaptoethanol (buffer A). Ten-ml fractions were collected and assayed for NAD-SDH activity. Fractions with activity were pooled, dialyzed against buffer A, and loaded on a column (1.5 cm i.d. × 2 cm) of DEAE-cellulose DE-52 (Whatman Co., Maidstone, England) that had been equilibrated with buffer A. The column was washed with buffer A and eluted with buffer A that contained 0.5 M KCl. Five-ml fractions were collected and NAD-SDH was assayed. The fractions with activity were pooled, dialyzed against buffer A, and loaded on a column (1.0 cm i.d. × 3.0 cm) of Blue Sepharose CL-6B (Pharmacia) that had been equilibrated with buffer A plus 2 mM DTT and 10% (v/v) glycerol (buffer B). The column was washed with buffer B, eluted with buffer B that contained 0.5 M KCl, and the eluate was discarded. The column was again washed with buffer B and then eluted with buffer B plus 30 mM NAD. One-ml fractions were collected and assayed for NAD-SDH activity. The fractions with activity were pooled, concentrated in a collodion bag (Sartorius AG., Göttingen, Germany), and loaded on an FPLC column (1.0 cm i.d. × 30.0 cm) of Superose 6 (Pharmacia) that had been equilibrated with buffer B. The column was eluted with the same buffer. Fractions of 0.3 ml were collected and assayed for NAD-SDH activity. The active fractions coinciding with the peak of the protein (absorbance at 280 nm) were pooled as purified NAD-SDH.

*Assays of enzymatic activity*—The activity of NAD-SDH was determined spectrophotometrically either by following the reduction of NAD in the presence of sorbitol or by following the oxidation of NADH in the presence of fructose at 340 nm. The reaction mixture (0.6 ml) contained 68 mM Tris-HCl (pH 9.0), 1 mM NAD, 400 mM sorbitol, and an aliquot of the preparation of NAD-SDH (for the reduction of NAD), or 62 mM Tris-acetate (pH 6.0), 0.05 mM NADH, 400 mM fructose, and an aliquot of the preparation of NAD-SDH (for the oxidation of NADH). All assays were performed at 25°C.

*Quantitation of protein*—Protein content was determined by the method of Read and Northcote (1981). Bovine serum albumin was used as the standard.

*SDS-PAGE*—SDS-PAGE on a 10% slab gel with a 4.5% stacking gel was performed by the method described by Laemmli (1970). Proteins were detected by staining with Coomassie brilliant blue R-250.

## Results and Discussion

*Purification of NAD-SDH*—The results of a typical purification are summarized in Table 1. Only one peak of NAD-SDH activity was detected at each chromatographic step. The enzyme was purified 158-fold and the recovery was 12% from the chromatography on Butyl-Toyopearl to that on Superose 6. The activity was barely detectable in the crude extract, perhaps because of the presence of some

**Table 1** Summary of the purification of NAD-SDH from 800 g of apple fruit

| Step | Activity[a] ($\mu$mol min$^{-1}$) | Protein (mg) | Specific activity ($\mu$mol min$^{-1}$ (mg protein)$^{-1}$) | Purification (-fold) |
|---|---|---|---|---|
| Crude extract | | 248 | | |
| Butyl-Toyopearl | 0.517 | 75.5 | 0.007 | 1.0 |
| DEAE-cellulose | 0.357 | 37.0 | 0.010 | 1.4 |
| Blue Sepharose | 0.159 | 0.220 | 0.723 | 103.3 |
| Superose 6 | 0.063 | 0.057 | 1.105 | 157.9 |

[a] The sorbitol-oxidation activity was estimated as described in Materials and Methods.

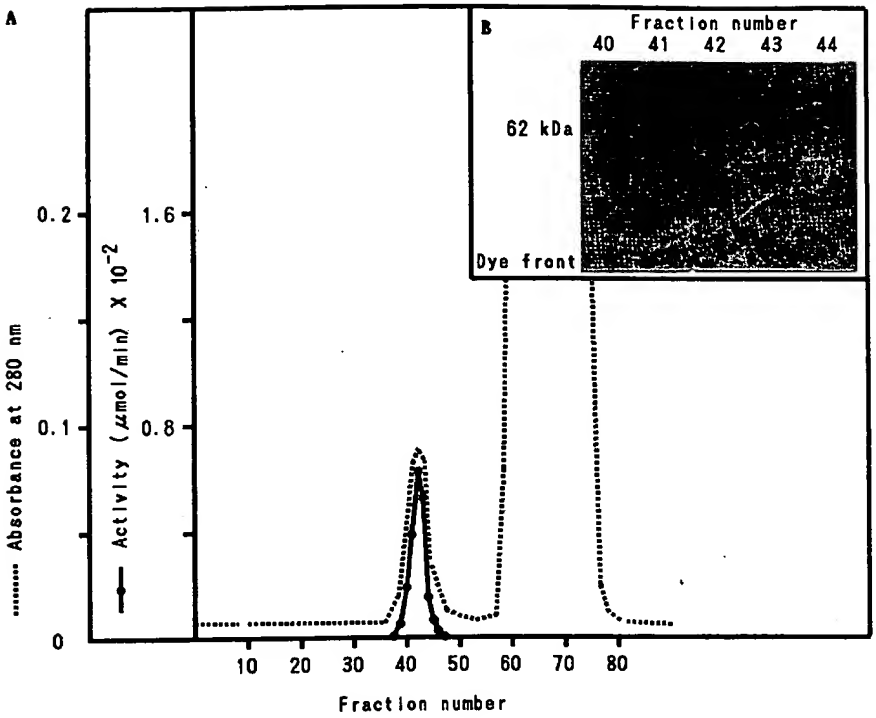


**Fig. 1** Column chromatography on Superose 6 of NAD-SDH. (A) Profiles of protein concentration and NAD-SDH activity. (B) SDS-polyacrylamide gel (10%) stained for protein with Coomassie brilliant blue R-250.

inhibitors of the enzyme in the initial homogenate. The specific activity of the purified enzyme was 1.11 μmol of sorbitol oxidized $min^{-1}$ (mg protein)$^{-1}$.

The profile of elution after FPLC on the column of Superose 6 is shown in Figure 1A. The highest activity of NAD-SDH was observed in fraction 42. The peak from fraction 57 to fraction 78 was β-NAD. SDS-PAGE of the preparation of enzyme in fractions 40 to 44 gave a single band. The intensity of stained bands on the gel corresponded to the strength of the activity of respective samples (Fig. 1B).

*Molecular mass of NAD-SDH and its subunit*—The molecular mass of NAD-SDH was estimated to be 120 kDa by gel filtration (Fig. 2). By cotrast, that of the NAD-SDH from maize endosperm was reported to be 78 kDa (Doehlert 1987). SDS-PAGE gave a single band of a peptide of 62 kDa (Fig. 3). Therefore, NAD-SDH appeared to be a homodimer.

*Properties of NAD-SDH*—NAD-SDH had different pH optima (Fig. 4) for the oxidation of sorbitol (pH 9.6) and the reduction of fructose (pH 6.0). The pH values are similar to those of the activities from apple callus tissue (Negm and Loescher 1979) and maize endosperm (Doehlert 1987).

The kinetics of the reactions catalyzed by NAD-SDH resembled Michaelis-Menten kinetics for both the oxidation of sorbitol and the reduction of fructose (Fig. 5). NAD-SDH had a $K_m$ of 40.3 mM for sorbitol. The reported values of the $K_m$ for sorbitol for the enzymes from

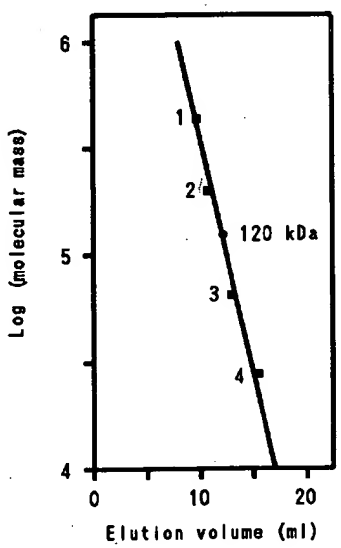


**Fig. 2** Estimation of the molecular mass of NAD-SDH by gel filtration on Superose 6 using standard protein markers: 1, apoferritin (443 kDa); 2, β-amylase (200 kDa); 3, bovine serum albumin (66 kDa); 4, carbonic anhydrase (29 kDa).

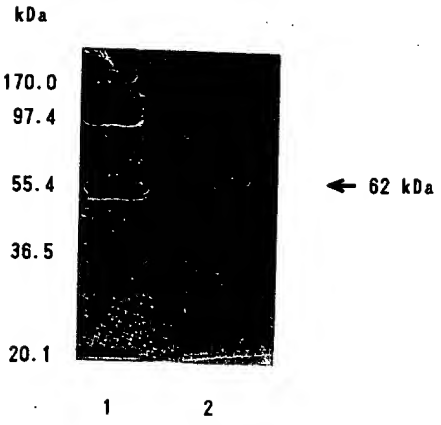


**Fig. 3** Determination of the molecular mass of NAD-SDH on an SDS-polyacrylamide gel (10%) stained for protein with Coomassie brilliant blue R-250. (1) Size markers. (2) NAD-SDH.

apple callus tissue (Negm and Loescher 1979) and maize endosperm (Doehlert 1987) were 86 mM and 8.45 mM, respectively. The maximum rate of oxidation of sorbitol was 1.31 $\mu$mol min$^{-1}$ (mg protein)$^{-1}$, which is lower than the rate of 5.87 $\mu$mol min$^{-1}$ (mg protein)$^{-1}$ reported for maize endosperm (Doehlert 1987). The $K_m$ value for fructose was 215 mM, while the corresponding $K_m$ values for the activity from apple callus tissue (Negm and Loescher 1979) and maize endosperm (Doehlert 1987) were 1.5 M and 136 mM, respectively. The maximum rate of reduction of fructose (0.12 $\mu$mol min$^{-1}$ (mg protein)$^{-1}$) was lower than the reported rate of 21.2 $\mu$mol min$^{-1}$ (mg protein)$^{-1}$ for the enzyme from maize endosperm (Doehlert 1987). The kinetic properties (Fig. 5), in particular the maximum rate of reduction of fructose, appeared to be different from those reported by Doehlert (1987). Doehlert (1987) reported that NAD-SDH from maize endosperm seemed to favor the conversion of fructose to sorbitol and suggested that the enzyme might function to metabolize some of the fructose produced from the degradation of the translocated sucrose. Hansen and Ryugo (1979) reported that, in prune fruit to which sorbitol is translocated, sorbitol was readily converted to other sugars. Our results (Fig. 5) suggest that in apple fruit also, in which sorbitol is the primary trans-

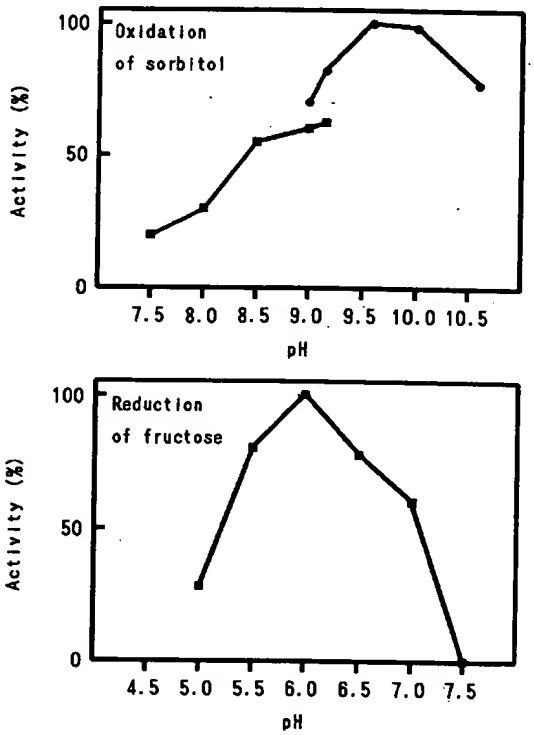


**Fig. 4** Effect of pH on the activity of NAD-SDH. The maximum rate of oxidation of sorbitol was 1.16 $\mu$mol min$^{-1}$ (mg protein)$^{-1}$ and that of reduction of fructose was 0.14 $\mu$mol min$^{-1}$ (mg protein)$^{-1}$. Reaction mixtures were as described in Materials and Methods with the exception that the buffers used were 50 mM glycine-NaOH from pH 9.0 to 10.6, 50 mM Tris-HCl from pH 7.5 to 9.2, and 50 mM Tris-acetate from pH 5.0 to 7.5.

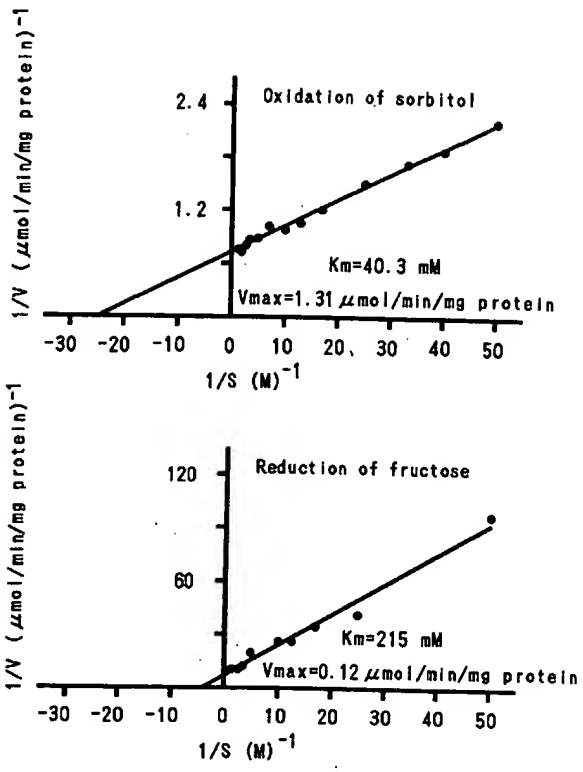


**Fig. 5** Double-reciprocal plots of reaction velocity *versus* the concentration of sorbitol or fructose for NAD-SDH. The composition of each reaction mixture was as described in Materials and Methods with the exception that the buffer used for the sorbitol-oxidation reaction was 68 mM glycine-NaOH (pH 9.6).

**Table 2** Effects of metal ions on the activity of NAD-SDH

| Salt | Activity[a] (%) |
|---|---|
| No additions | 100 |
| 1 mM $BaCl_2$ | 87 |
| 1 mM $CaCl_2$ | 72 |
| 10 mM $CaCl_2$ | 71 |
| 1 mM $MgCl_2$ | 54 |
| 10 mM $MgCl_2$ | 54 |
| 1 mM $ZnCl_2$ | 22 |
| 1 mM $HgCl_2$ | 9 |

[a] The sorbitol-oxidation activity (the results are averages from 4 experiments) was estimated as described in Materials and Methods with the exception that the buffer used for the sorbitol-oxidation reaction was 68 mM glycine-NaOH (pH 9.6).

**Table 3** Substrate specificity of NAD-SDH

| Substrate | Activity[a] (%) |
|---|---|
| 400 mM Sorbitol | 100 |
| 400 mM Xylitol | 40 |
| 400 mM L-Threitol | 36 |
| 400 mM D-Mannitol | 8 |
| 400 mM Ribitol | 7 |
| 400 mM L-Arabitol | 5 |
| 400 mM Ethyl alcohol | 51 |

[a] The oxidation of some substrates (the results are averages from 4 experiments) was estimated as described in Materials and Methods with the exception that the buffer used for the sorbitol-oxidation reaction was 68 mM glycine-NaOH (pH 9.6).

located product of photosynthesis (Webb and Burley 1962, Bieleski 1969, 1982, Chong and Taper 1971, Loescher 1987), NAD-SDH would favor the conversion of sorbitol to fructose. The NAD-SDH in apple fruit might be different in terms of its metabolic function from that in maize endosperm.

*Effects of divalent cations*—None of the tested divalent cations had an activating effect on the oxidation of sorbitol by NAD-SDH (Table 2), as also reported by Doehlert (1987) for the NAD-SDH from maize endosperm. NAD-SDH from apple fruit is similar to the enzyme from maize endosperm in this respect. Zinc ions have been reported to reverse inhibition by cysteine (Negm and Loesher 1979). However, NAD-SDH was rather strongly inhibited by zinc ions. Calcium and magnesium ions also inhibited the oxidation of sorbitol by NAD-SDH.

*Substrate specificity*—The substrate specificity of NAD-SDH was examined and the reaction catalyzed by the enzyme proved not to be specific to sorbitol (Table 3). NAD-SDH from apple callus tissue (Negm and Loescher 1979) and from maize endosperm (Doehlert 1987) were also reported to oxidize some substrates other than sorbitol. Xylitol and L-threitol had a relatively high affinity for NAD-SDH (Table 3), as also reported by Negm and Loescher (1979) and Doehlert (1987). D-Mannitol and ribitol were oxidized at a comparatively low rate, just as reported by Negm and Loescher (1979). L-Arabitol was oxidized by NAD-SDH at a low rate (5% of the rate of oxidation of sorbitol), and the enzyme is similar in this respect to the enzyme from maize endosperm (Doehlert 1987) rather than to that from apple callus (Negm and Loescher 1979). Glycerol was not oxidized by NAD-SDH. The properties of NAD-SDH isolated from some animal systems have been reviewed and its close structural relationship to alcohol dehydrogenase has been discussed (Jeffery and Jörnvall 1988). Ethyl alcohol had a high affinity for NAD-SDH from apple fruit (51% of that of sorbitol). Further investigations are required to elucidate the structural, functional, and metabolic relationships between NAD-SDH and alcohol dehydrogenase in plant systems.

The present study yielded three prominent results. First, NAD-SDH was purified from plant tissue for the first time (Fig. 1A, Fig. 1B). Second, from the estimations of molecular mass (Fig. 2, Fig. 3) and its kinetic properties (Fig. 5), NAD-SDH from apple fruit appeared to be different from NAD-SDH from maize endosperm (Doehlert 1987), though some similarities were found in the effects of pH (Fig. 4) and divalent cations (Table 2), as well as in the substrate specificity (Table 3). Third, the kinetic properties (Fig. 5) suggested that the enzyme would favor the conversion of sorbitol to fructose, providing further evidence for the importance of the enzyme in the metabolism of the translocated sorbitol in apple fruit.

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